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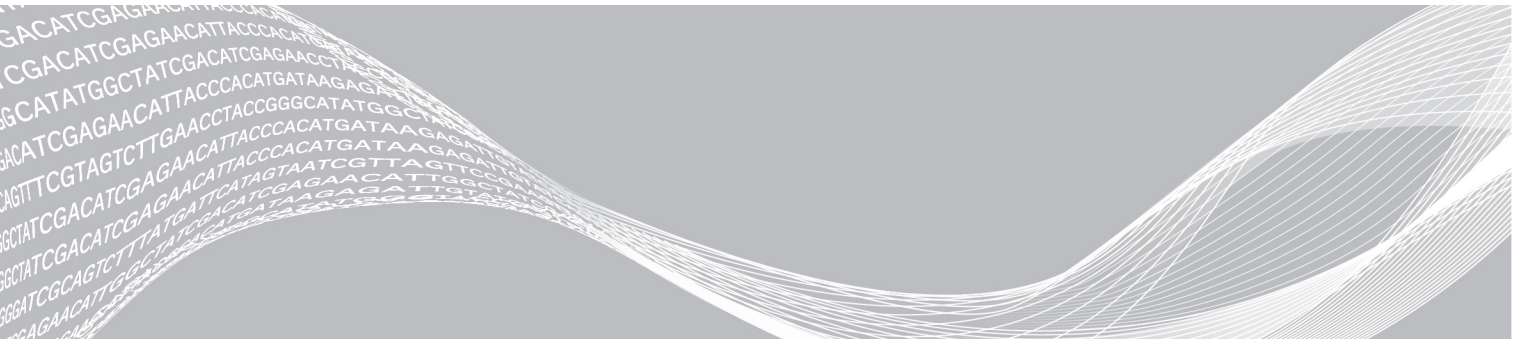
TruSight Oncology 500 High Throughput

Reference Guide



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Chapter 1 Overview

Introduction

The TruSight™ Oncology 500 High Throughput (HT) protocol describes an enrichment-based approach to convert DNA and RNA extracted from formalin-fixed paraffin embedded (FFPE) tissue samples into libraries enriched for cancer-related genes that can be sequenced on Illumina® sequencing systems. TruSight Oncology 500 HT enables the preparation of 144 libraries from DNA, or a combination of DNA and RNA libraries.

The kit is optimized to provide high sensitivity and specificity for low-frequency somatic variants across 523 genes. DNA biomarkers include the following:

- Single nucleotide variants (SNVs)
- Insertions
- Deletions
- Gene amplifications
- Multinucleotide variants (MNVs)

TruSight Oncology 500 HT also detects immunotherapy biomarkers for tumor mutational burden (TMB) and microsatellite instability (MSI) in DNA. Fusions and splice variants are detected in RNA.

DNA/RNA Input Recommendations

Use a minimum of 40 ng DNA input and 80 ng RNA input with the TruSight Oncology 500 HT assay. Inputs lower than those specified can decrease library yield and quality. Quantify the input nucleic acids before beginning the protocol. To obtain sufficient nucleic acid material, isolate nucleic acid from a minimum of 2 mm^3 of FFPE tissue. Additionally, follow these guidelines for input:

- Use a nucleic acid isolation method that produces high recovery yields, minimizes sample consumption, and preserves sample integrity. The QIAGEN AllPrep DNA/RNA FFPE Kit provides a high yield of nucleic acids.
- For recommendations for obtaining sufficient nucleic acid material, see the TruSight Oncology 500 High Throughput support page on the Illumina support website.
- Use a fluorometric quantification method that uses DNA/RNA binding dyes such as AccuClear™ (DNA) or QuantiFluor® (RNA).

Compatibility

For information on sequencing compatibility and run settings, see the TruSight Oncology 500 HT support pages on the Illumina website.

Sample Qualification

For optimal performance, assess DNA and RNA sample quality before using the TruSight Oncology 500 HT assay.

- DNA samples can be assessed using the Illumina FFPE QC Kit.
- Use DNA samples that result in a delta Cq value ≤ 5. Samples with a delta Cq > 5 may result in decreased assay performance.
- RNA samples can be assessed using Advanced Analytical Technologies Fragment Analyzer™ (Standard Sensitivity RNA Analysis Kit) or Agilent Technologies 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit).
- Use RNA samples that result in a DV_{200} value of ≥ 20%. Using samples with a DV_{200} value < 20% may result in decreased assay performance.

Reference Samples (Optional)

- Use reference materials with known variant composition, such as Horizon Discovery HD753 (DNA) and Agilent Universal Human Reference RNA. The Agilent Universal Human Reference RNA is an intact RNA sample. Process the sample after the intact RNA procedure described in *Denature and Anneal RNA* on page 10.
- Use RNase/DNase-free water as a no template control. Do not sequence the no template control.
- Processing a reference sample or no template control reduces the total number of test samples that can be processed.

DNA Shearing Recommendations

The TruSight Oncology 500 HT assay is optimized to prepare libraries from gDNA that are fragmented to 90-250 bp. The assay is optimized using the Covaris E220*evolution*™, LE220, or ME220 Focused-ultrasonicator with the parameters provided in *Fragment gDNA* on page 14. Fragment size distribution can vary due to differences in sample quality and the sonication instrumentation used for fragmentation.

- **[Optional]** Assess fragment size distribution of sheared samples using the Agilent DNA 1000 Kit with the Agilent Bioanalyzer 2100.

Additional Resources

The TruSight Oncology 500 HT support pages on the Illumina website provide software, training resources, product compatibility information, and the following documentation. Always check support pages for the latest versions.

Chapter 2 Protocol

Introduction

This section describes the TruSight Oncology 500 HT protocol.

- Review the complete sequencing workflow, from sample through analysis, to ensure compatibility of products and experiment parameters.
- Before proceeding, confirm kit contents and make sure that you have the required consumables and equipment.
  - TruSight Oncology 500 HT kits do not include index anchors or indexes.
- Follow the protocol in the order described using the specified parameters.
- Before beginning library preparation, record sample information, and assign each sample a unique index.

Prepare for Pooling

If you plan to pool libraries, record information about your samples before starting library prep. Use a recording tool that is compatible with your sequencing system and library information. For compatibility information, see the TruSight Oncology 500 HT support page on the Illumina website or the support pages for your system.

- For strategies on forming low plex, color-balanced pools, see the *Index Adapters Pooling Guide (document # 1000000041074)*.
- For index adapter sequences and how to record them, see *Illumina Adapter Sequences (document # 1000000002694)*.

Tips and Techniques

Protocol Continuity

- Review tips and techniques before starting the protocol, as many critical techniques are listed only here and are not repeated in the protocol.
- Follow the protocol in the order described using the specified parameters.
- Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

Avoiding Cross-Contamination

- When adding or transferring samples or reagents, change tips between ***each well***.
- Use a unidirectional workflow when moving from pre-amp to post-amp areas.
- To prevent amplification product or probe carryover, avoid returning to the pre-amp area after beginning work in the post-amp area.
- When adding indexing primers, change tips between ***each well***.
- Change gloves if gloves come in contact with indexing primers, samples, or probes.
- Clean work surfaces and equipment thoroughly before and after the procedure with an RNase/DNase inhibiting cleaner.

Sealing the Plate

- Always seal the plate before the following steps in the protocol:
  - Shaking steps
  - Vortexing steps
  - Centrifuge steps
  - Thermal cycling steps
  - Heated incubation steps
  - Long term storage
- Apply the adhesive seal to cover the plate and seal with a rubber roller, making sure edges and wells are completely sealed.
- Apply a new seal every time you cover a plate.
- Use adhesive seals for shaking, vortexing, centrifuging, thermal cycling, heated incubations, and long-term storage. The seals are effective at -40°C to 110°C and suitable for skirted or semiskirted PCR plates.
- If you observe droplets hanging from the inside of a sealed plate, centrifuge at 280 x g for 1 minute.

Plate Transfers

- When transferring volumes between plates, transfer the specified volume from each well of a plate to the corresponding well of the other plate.

Centrifugation

- When instructed to centrifuge the plate, centrifuge at 280 x g for 1 minute.

Handling Reagents

- Tightly recap all reagent tubes immediately after use to limit evaporation and prevent contamination.
- Return reagents to the recommended storage conditions when they are no longer needed for the procedure.
- Stability of the TruSight Oncology 500 HT Kit has been evaluated and performance demonstrated for up to four uses of the kit.
- Master mix preparation tables include volume overage to ensure that there is sufficient volume per sample.

Handling Beads

- Do not freeze beads.
- Pipette bead suspensions slowly.
- Before use, allow the beads to come to room temperature.
- Mix beads for 1 minute to ensure homogeneity.
- If beads are aspirated into pipette tips, dispense back to the plate on the magnetic stand, and wait until the liquid is clear (~2 minutes).
- When washing beads:
  - Use the specified magnetic stand for the plate.
  - Dispense liquid so that beads on the side of the wells are wetted.
  - Keep the plate on the magnetic stand until the instructions specify to remove it.
  - Do not agitate the plate while it is on the magnetic stand. Do not disturb the bead pellet.
- When mixing beads with a pipette:
  - Use a suitable pipette and tip size for the volume you are mixing (for example, use a P200 for volumes from 20 µl to 200 µl).
  - Adjust the volume setting to ~50-75% of your sample volume.
  - Pipette with a slow, smooth action.
  - Avoid aggressive pipetting, splashing, and introducing bubbles.
  - Position the pipette tip above the pellet and dispense directly into the pellet to release beads from the well or tube.
  - Make sure that the bead pellet is fully in solution. (For example, for SMB pellets, the solution should look dark brown and have a homogenous consistency.)

Library Prep DNA Only Workflow

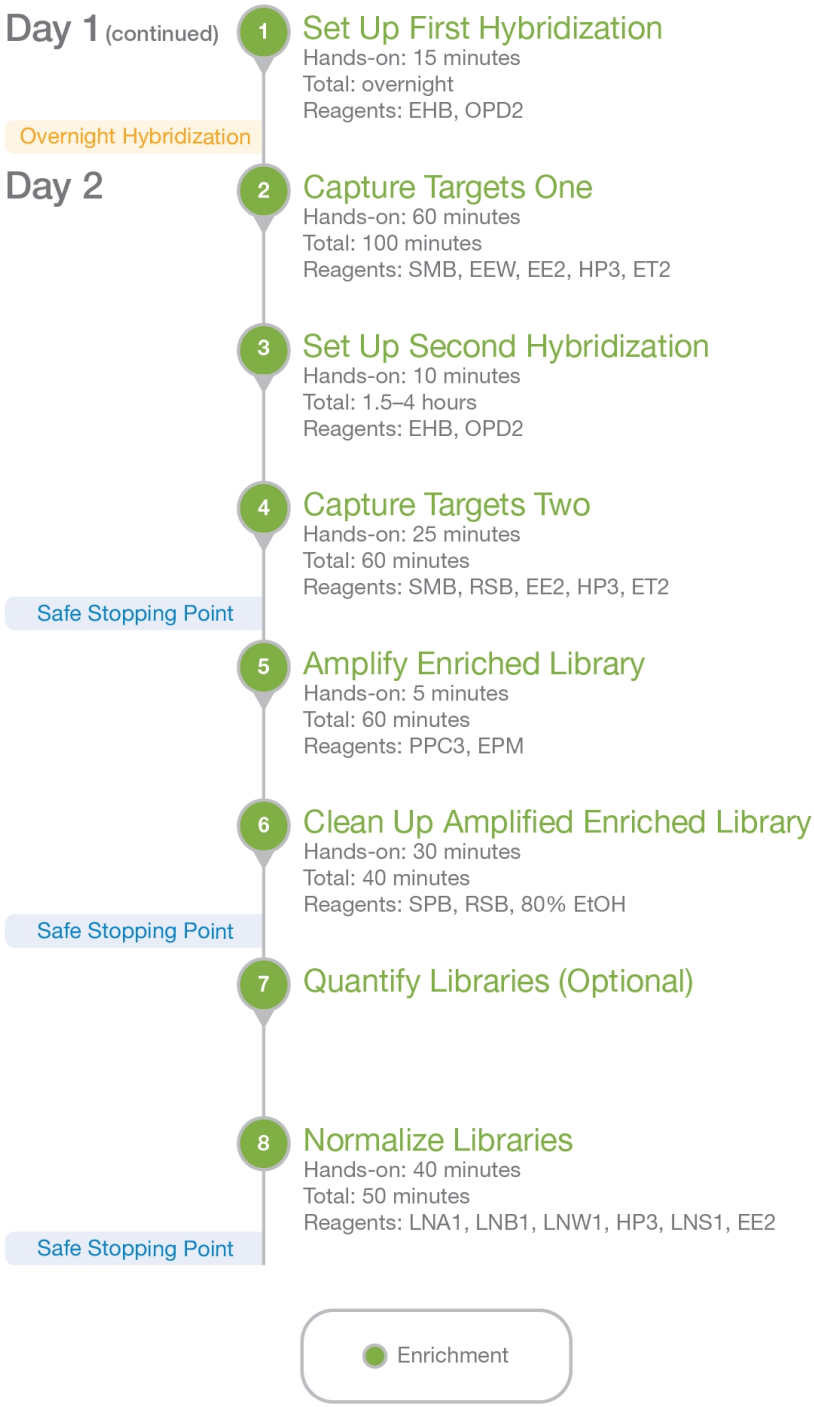
The following diagram illustrates the recommended DNA only library preparation workflow using a TruSight Oncology 500 HT kit. Safe stopping points are marked between steps. Hands-on and total times are approximate.

Figure 1 TruSight Oncology 500 High Throughput DNA Only Workflow (Part 1)

Enrichment DNA Only Workflow

The following diagram illustrates the recommended DNA only enrichment workflow using a TruSight Oncology 500 HT kit. Safe stopping points are marked between steps. Hands-on and total times are approximate .

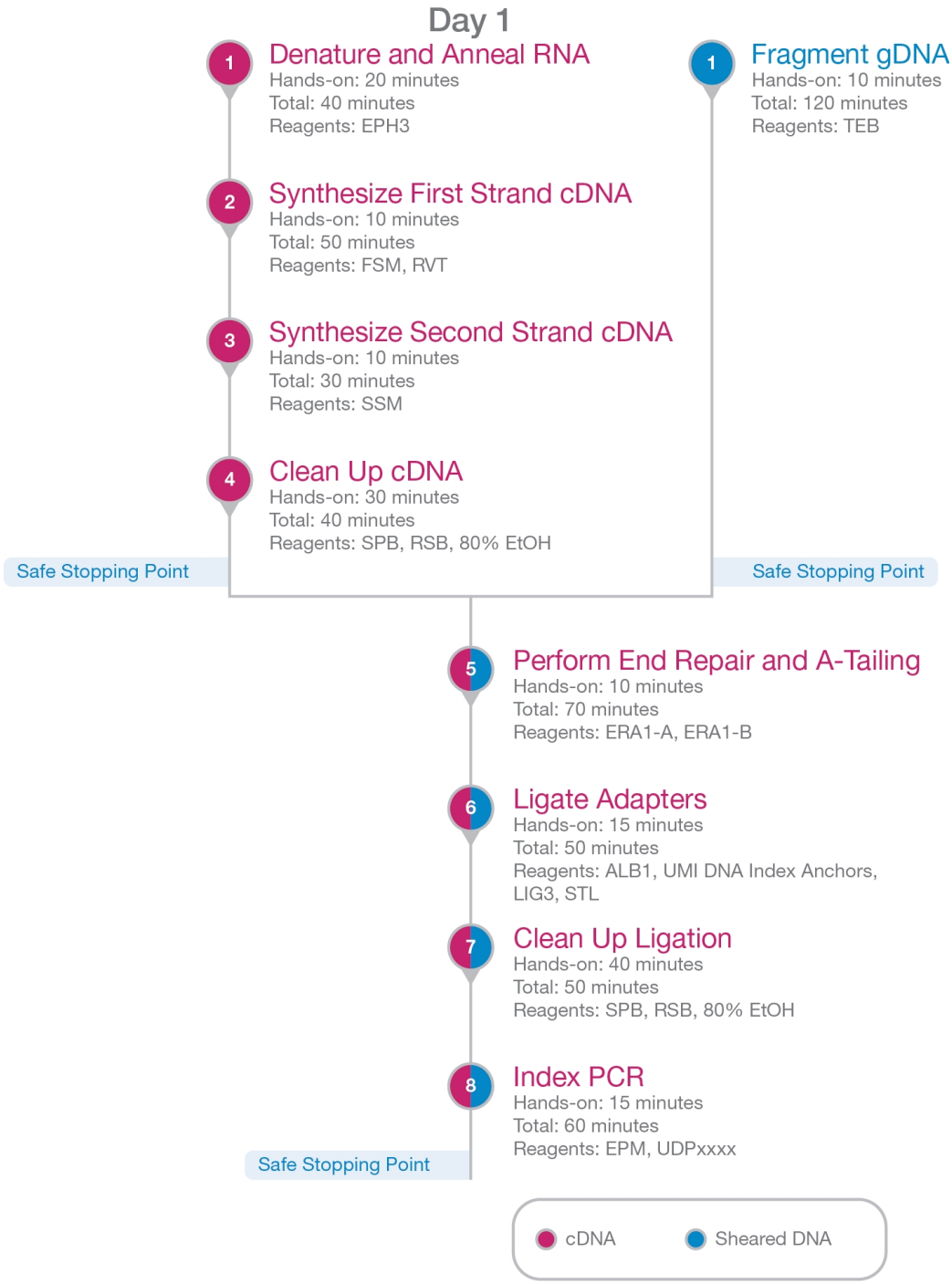
Figure 2 TruSight Oncology 500 High Throughput DNA Only Workflow (Part 2)



Library Prep DNA and RNA Workflow

The following diagram illustrates the recommended DNA and RNA library preparation workflow using a TruSight Oncology 500 HT kit. RNA and DNA libraries can be prepared simultaneously. Safe stopping points are marked between steps. Hands-on and total times are approximate.

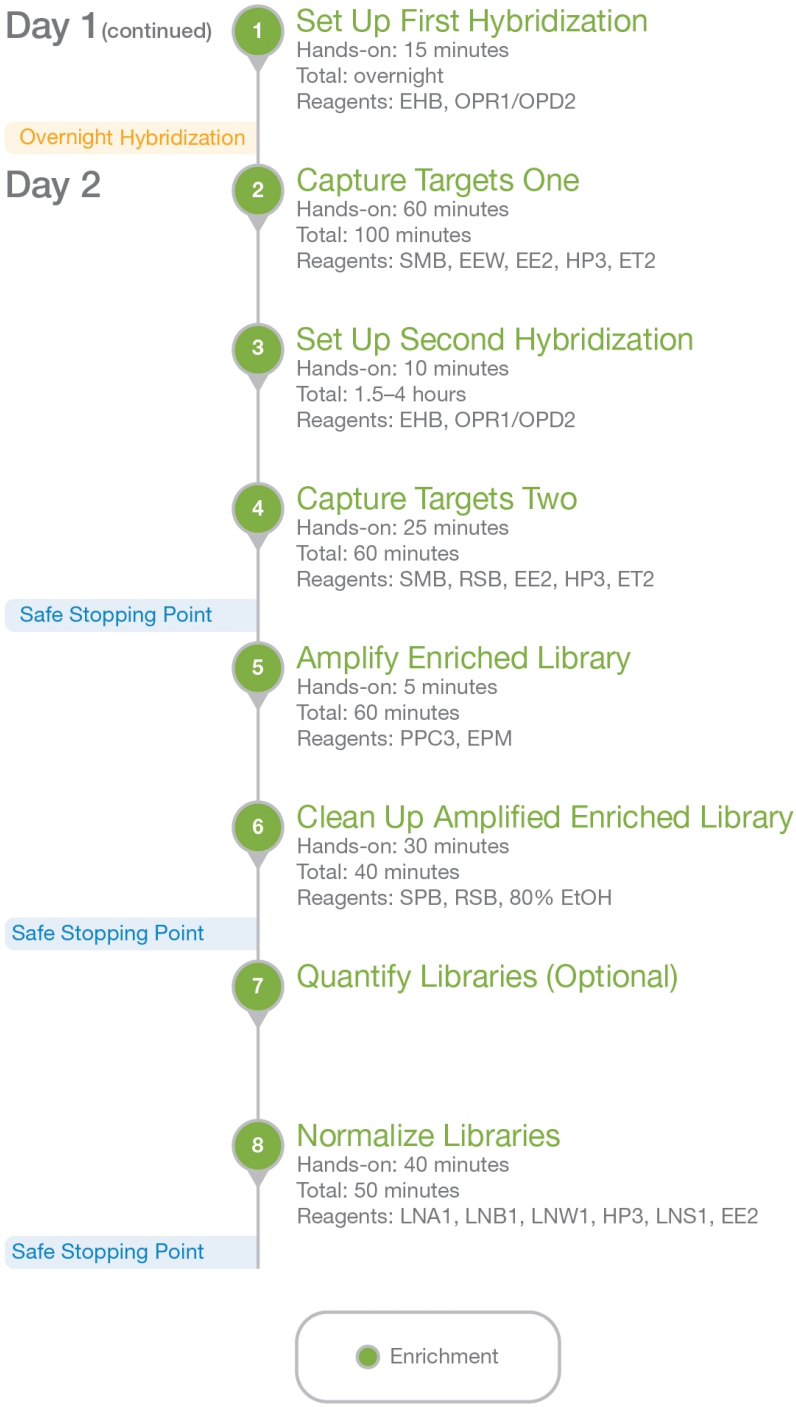
Figure 3 TruSight Oncology 500 High Throughput DNA and RNA Workflow (Part 1)



Enrichment DNA and RNA Workflow

The following diagram illustrates the recommended DNA and RNA enrichment workflow using a TruSight Oncology 500 HT kit. Safe stopping points are marked between steps. Hands-on and total times are approximate.

Figure 4 TruSight Oncology 500 High Throughput DNA and RNA Workflow (Part 2)



Denature and Anneal RNA

This process denatures purified RNA and primes the RNA with random hexamers in preparation for cDNA synthesis.

Consumables

- EPH3 (Elution, Primer, Fragmentation Mix)
- FSM (First Strand Synthesis Mix)
- RVT (Reverse Transcriptase)
- Nuclease-free water
- 96-well PCR plate
- 1.7 mL microcentrifuge tube
- Microseal 'B' adhesive seals

Preparation

1 Prepare the following consumables:

Reagent	Storage	Instructions
EPH3	-25°C to -15°C	Thaw to room temperature. Vortex to resuspend. Centrifuge briefly.
FSM	-25°C to -15°C	Thaw to room temperature. Vortex to resuspend. Centrifuge briefly.
RVT	-25°C to -15°C	Keep on ice. Centrifuge briefly and then pipette to mix.

2 Thaw extracted RNA samples on ice.

3 Qualify and quantify the samples. See *DNA/RNA Input Recommendations* on page 1.

4 Pipette each RNA sample 10 times to mix and then centrifuge briefly.

5 Use RNase/DNase-free water to prepare a minimum of 80 ng of each purified RNA sample in a final volume of 8.5 µl (9.4 ng/µl).

6 Pipette to mix prepared RNA samples and then centrifuge briefly.

7 Set prepared RNA samples on ice.

8 Label the 96 well PCR plate CF (cDNA fragments)

9 Save the following programs on the thermal cycler:
   - For FFPE or fragmented RNA, save the LQ-RNA program.
     - Choose the preheat lid option and set to 100°C
     - Set the reaction volume to 17 µl
     - 65°C for 5 minutes
     - Hold at 4°C
   - For cell line or intact RNA, save the HQ-RNA program.
     - Choose the preheat lid option and set to 100°C
     - Set the reaction volume to 17 µl
     - 94°C for 8 minutes
     - Hold at 4°C

Procedure

1 Combine the appropriate volumes from the table below in a microcentrifuge tube to prepare the FSM+RVT Master Mix.

Master Mix Component	8 Samples (µl)	24 Samples (µl)	48 Samples (µl)	72 Samples (µl)
FSM	72	216	432	648
RVT	8	24	48	72

2 Pipette 10 times to mix and then place on ice until *Synthesize First Strand cDNA* on page 11.

3 Add 8.5 µl of each RNA sample (9.4 ng/µl) to a unique well of the CF PCR plate.

4 Add 8.5 µl EPH3 to each well.

5 Apply Microseal 'B' to the CF PCR plate and shake the plate at 1200 rpm for 1 minute.

6 Centrifuge the plate at 280 × g for 1 minute.

7 Place the plate on the preprogrammed thermal cycler and run the LQ-RNA or HQ-RNA program.

Synthesize First Strand cDNA

This process reverse transcribes the RNA fragments primed with random hexamers into first strand cDNA using reverse transcriptase.

Consumables

- FSM+RVT Master Mix
- Microseal 'B' adhesive seals

Preparation

1 Save the following 1stSS program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 25 µl
   - 25°C for 10 minutes
   - 42°C for 15 minutes
   - 70°C for 15 minutes
   - Hold at 4°C

Procedure

1 Remove the CF PCR plate from the thermal cycler.

2 Pipette to mix FSM+RVT Master Mix.

3 Add 8 µl FSM+RVT Master Mix to each well.

4 Discard any remaining master mix after use.

5 Pipette to mix 5 times.

6 Apply Microseal 'B' to the CF PCR plate and shake the plate at 1200 rpm for 1 minute.

7 Centrifuge the plate at 280 × g for 1 minute.

8 Place the plate on the preprogrammed thermal cycler and run the 1stSS program.

Synthesize Second Strand cDNA

This process removes the RNA template and synthesizes double-stranded cDNA.

Consumables

- SSM (Second Strand Mix)
- Microseal 'B' adhesive seals

Preparation

1 Prepare the following consumables:

Reagent	Storage	Instructions
SSM	-25°C to -15°C	Thaw to room temperature. Invert 10 times to mix. Centrifuge briefly.

2 Save the following 2ndSS program on the thermal cycler with a heated lid.

NOTE

If the lid temperature cannot be set to 30°C, turn off the preheated lid heat option.

- Choose the preheat lid option and set to 30°C
- Set the reaction volume to 50 µl
- 16°C for 25 minutes
- Hold at 4°C

Procedure

1 Remove the CF PCR plate from the thermal cycler.

2 Add 25 µl SSM to each well.

3 Apply Microseal 'B' to the CF PCR plate and shake the plate at 1200 rpm for 1 minute.

4 Place the plate on the preprogrammed thermal cycler and run the 2ndSS program.

Clean Up cDNA

This process uses SPB to purify the cDNA from unwanted reaction components. The beads are washed twice with fresh 80% ethanol and the cDNA is eluted with RSB.

Consumables

- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- Freshly prepared 80% ethanol (EtOH)
- 96-well MIDI plate
- **[Optional]** 96-well PCR plate
- Microseal 'B' adhesive seals

About Reagents

- Aspirate and dispense SPB slowly due to the viscosity of the solution.

Preparation

1 Prepare the following consumables:

Reagent	Storage	Instructions
RSB	2°C to 8°C or -25°C to -15°C	Bring to room temperature. If stored at -25°C to -15°C, thaw at room temperature and vortex before use.
SPB	2°C to 8°C	Bring to room temperature for at least 30 minutes.

2 Label a new 96-well MIDI plate BIND1.

3 Select one of the following plate options.
- Label a new 96-well MIDI plate PCF (Purified cDNA Fragments) to continue with library prep immediately after cleaning up cDNA.
- Label a new 96 well PCR plate PCF (Purified cDNA Fragments) to store the plate after cleaning up cDNA.

4 Prepare fresh 80% EtOH.

Procedure

Bind

1 Remove the CF PCR plate from the thermal cycler.

2 Vortex SPB for 1 minute to resuspend the beads.

3 Add 90 µl SPB to each well of the BIND1 MIDI plate.

4 Transfer 50 µl of each sample from the CF PCR plate to the corresponding well of the BIND1 MIDI plate.

5 Apply Microseal 'B' to the BIND1 MIDI plate and shake the plate at 1800 rpm for 2 minutes.

6 Incubate at room temperature for 5 minutes.

7 Place the BIND1 MIDI plate on a magnetic stand for 5 minutes.

8 Use a pipette to remove and discard all supernatant from each well.

Wash

1 Wash beads as follows.

a Keep on magnetic stand and add 200 µl fresh 80% ethanol to each well.
b Wait 30 seconds.
c Remove and discard all supernatant from each well.

2 Repeat step 1 to wash beads a **second** time.

3 Use a P20 pipette with fine tips to remove residual supernatant from each well.

Elute

1 Remove the BIND1 MIDI plate from the magnetic stand.

2 Add 22 µl RSB to each well.

3 Apply Microseal 'B' to the BIND1 MIDI plate and shake the plate at 1800 rpm for 2 minutes.

4 Incubate at room temperature for 2 minutes.

5 Place on a magnetic stand for 2 minutes.

6 Transfer 20 µl eluate from each well of the BIND1 MIDI plate to the corresponding well of the PCF plate.

7 Add 30 µl RSB to each sample well of the PCF plate, and then pipette at least 10 times to mix.

8 Apply Microseal 'B' to the PCF plate.

SAFE STOPPING POINT

If you are stopping, ensure that the PCF PCR plate is sealed, and briefly centrifuge at 280 × g. Store at -25°C to -15°C for up to 7 days.

Fragment gDNA

This process fragments gDNA and generates 90-250 bp dsDNA fragments with 3' or 5' overhangs using a Covaris ultrasonicator.

Consumables

- TEB (TE Buffer)
- Covaris 8 microTUBE Strip with foil seals
- 96-well MIDI plate
- **[Optional]** 96-well PCR plate

Preparation

1 Prepare the following consumables:

Reagent	Storage	Instructions
TEB	2°C to 8°C	Bring to room temperature. Vortex to mix.

2 Turn on and set up the Covaris instrument according to manufacturer guidelines.

NOTE
The instrument can take approximately one hour to de-gas.

3 Choose one of the following plate options:
- Label a new 96 well MIDI plate LP (Library Preparation) if proceeding with library prep immediately after shearing gDNA.
- Label a new 96 well PCR plate LP (Library Preparation) to store the plate after shearing gDNA.

4 Thaw gDNA samples at room temperature.

5 Refer to *DNA/RNA Input Recommendations* on page 1 to qualify and quantify samples.

6 Pipette each gDNA sample 10 times to mix and then centrifuge briefly.

7 Use TEB to prepare a minimum of 40 ng of each purified gDNA sample in a final volume of 12 µl (3.3 ng/µl).

Procedure

1 Add 12 µl of each prepared gDNA sample into a separate well of a Covaris 8 microTUBE Strip.

2 Add 40 µl TEB to each sample.

3 Pipette to mix.

NOTE

Avoid introducing excessive bubbles or air gaps in the shearing tube as incomplete shearing may result.

4 Fill any unused Covaris 8 microTUBE Strip wells with 52 µl water.

5 Seal the microTUBE Strip with the foil seal.

6 Centrifuge briefly.

7 If you are using the Covaris E220 *evolution*, LE220, or ME220 model, fragment the gDNA using the following settings.

Setting	E220*evolution*	LE220	ME220
Peak Incident Power	175 watts	450 watts	50 watts
Duty Factor	10%	30%	30%
Cycles per Burst	200	200	1000
Treatment Time	280 seconds	250 seconds	10 seconds
Temperature	7°C	7°C	12°C
Intensifier	yes	N/A	N/A
Other	Intensifier	N/A	Wave guide
Pulse repeats	N/A	N/A	20
Average power	N/A	N/A	15 watts

8 Centrifuge tube strip briefly to collect droplets.

9 Transfer 50 µl of each sheared gDNA sample to an empty well of the LP plate.

- A P20 pipette with fine tips can be used when transferring sheared gDNA sample to the LP plate (pipette 20 µl + 20 µl + 10 µl).

SAFE STOPPING POINT

If you are stopping, apply Microseal 'B' to the LP PCR plate and briefly centrifuge at 280 × g. Store at -25°C to -15°C for up to 7 days.

Perform End Repair and A-Tailing

This process converts the overhangs resulting from fragmentation into blunt ends using an End Repair A-Tailing master mix (ERA1). The 3' to 5' exonuclease activity of this mix removes the 3' overhangs and the 5' to 3' polymerase activity fills in the 5' overhangs. The 3' ends are A-tailed during this reaction to prevent them from ligating to each other during the adapter ligation reaction.

Consumables

- ERA1-A (End Repair A-tailing Enzyme Mix 1)
- ERA1-B (End Repair A-tailing Buffer 1)
- 1.7 ml microcentrifuge tube
- 96-well MIDI plate
- Microseal 'B' adhesive seals

Preparation

1 Prepare the following consumables.

Reagent	Storage	Instructions
ERA1-A	-25°C to -15°C	Keep on ice. Centrifuge briefly, and then pipette to mix.
ERA1-B	-25°C to -15°C	Thaw to room temperature. Centrifuge briefly, and then pipette to mix. If precipitates are present, warm the tube in your hands, and then pipette to mix until the crystals dissolve.

2 Bring sheared gDNA and/or any cDNA to room temperature.

3 If the PCF or LP PCR plates were stored at -25° to -15°C, thaw at room temperature, pipette to mix, and then centrifuge.

4 Transfer 50 µl of each cDNA and/or sheared gDNA sample from the PCR plate to unique wells of a new 96-well MIDI plate.

5 Label the MIDI plate LP2 (Library Preparation 2).

6 Preheat two Hybex incubators with MIDI heat block inserts as follows.
- Preheat the first incubator to 30°C.
- Preheat the second incubator to 65°C.

7 Prepare an ice bucket.

Procedure

1 Combine the appropriate volumes from the table below in a microcentrifuge tube to prepare ERA1 Master Mix.

Master Mix Component	8 Samples (µl)	24 Samples (µl)	48 Samples (µl)	72 Samples (µl)
ERA1-B	69	207	415	622
ERA1-A	27	81	161	242

2 Pipette 10 times to mix, and then place ERA1 Master Mix on ice.

3 Add 10 µl ERA1 Master Mix to each sample in the LP2 MIDI plate.

4 Discard any remaining master mix after use.

5 Apply Microseal ‘B’ to the LP2 MIDI plate and shake the plate at 1800 rpm for 2 minutes.

6 Incubate at 30°C for 30 minutes.

7 Immediately transfer to another incubator at 65°C and incubate for 20 minutes.

8 Place the LP2 MIDI plate on ice for 5 minutes.

Ligate Adapters

This process ligates adapters to the ends of the cDNA and/or gDNA fragments. UMI DNA index anchors (adapters) that contain unique molecular identifiers are ligated to both cDNA and gDNA fragments.

Consumables

- ALB1 (Adapter Ligation Buffer 1)
- LIG3 (DNA Ligase 3)
- STL (Stop Ligation Buffer)
- UMI DIA (Unique Molecular Identifier DNA Index Anchors)
- Microseal 'B' adhesive seals

About Reagents

- ALB1 is highly viscous. Pipette slowly to avoid forming bubbles.

Preparation

1 Prepare the following consumables:

Item	Storage	Instructions
ALB1	-25°C to -15°C	Thaw to room temperature. Vortex ≥ 10 seconds to resuspend. Centrifuge briefly.
LIG3	-25°C to -15°C	Keep on ice. Centrifuge briefly, and then pipette to mix.
STL	-25°C to -15°C	Thaw and bring to room temperature. Vortex to resuspend. Centrifuge briefly.
UMI DIA	-25°C to -15°C	Thaw to room temperature. Centrifuge briefly.

NOTE

Each well of the UMI DIA plate contains enough volume for one use only.

Procedure

1 Add 60 µl ALB1 to each well.

2 Add 5 µl LIG3 to each well.

3 Pipette UMI DIA to mix. Pierce the foil seal on the UMI DIA plate with a new pipette tip for each well for only the number of samples being processed.

4 Add 10 µl UMI DIA to each well.

5 Apply Microseal 'B' to the LP2 MIDI plate and shake the plate at 1800 rpm for 2 minutes.

6 Incubate at room temperature for 30 minutes.

7 Add 5 µl STL to each well.

8 Apply Microseal 'B' to the LP2 MIDI plate and shake the plate at 1800 rpm for 2 minutes.

Clean Up Ligation

This process uses SPB to purify the gDNA and cDNA fragments and remove unwanted products, such as unligated adapters. The beads are washed twice with fresh 80% ethanol and the product is eluted with RSB.

Consumables

- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- Freshly prepared 80% ethanol (EtOH)
- Microseal 'B' adhesive seals
- 96-well PCR plate

About Reagents

- Aspirate and dispense SPB slowly due to the viscosity of the solution.

Preparation

1 Prepare the following consumables:

Reagent	Storage	Instructions
RSB	2°C to 8°C -25°C to -15°C	Bring to room temperature. If stored at -25°C to -15°C, thaw to room temperature and vortex before use.
SPB	2°C to 8°C	Bring to room temperature for at least 30 minutes.

2 Label a new 96-well PCR plate LS (Library Samples).

3 Prepare fresh 80% EtOH.

Procedure

Bind

1 Vortex SPB for 1 minute to resuspend the beads.

2 Add 112 µl SPB to each well of the LP2 MIDI plate.

3 Apply Microseal 'B' to the LP2 MIDI plate and shake at 1800 rpm for 2 minutes.

4 Incubate at room temperature for 5 minutes.

5 Place the LP2 MIDI plate on the magnetic stand for 10 minutes.

6 Use a pipette to remove and discard all supernatant from each well.

Wash

1 Wash beads as follows.

   a Keep on magnetic stand and add 200 µl fresh 80% ethanol to each well.
   b Wait 30 seconds.

c Remove and discard all supernatant from each well.

2 Repeat step 1 to wash beads a **second** time.

3 Use a P20 pipette with fine tips to remove residual supernatant from each well.

Elute

1 Remove the LP2 MIDI plate from the magnetic stand.

2 Add 22.5 µl RSB to each well.

3 Apply Microseal 'B' to the LP2 MIDI plate and shake the plate at 1800 rpm for 2 minutes.

4 Incubate at room temperature for 2 minutes.

5 Place on a magnetic stand for 2 minutes.

6 Transfer 20 µl of each eluate from the LP2 MIDI plate to the corresponding well of the LS PCR plate.

Index PCR

In this step, library fragments are amplified using primers that add index sequences for sample multiplexing. The resulting product contains the complete library of cDNA and DNA fragments flanked by index sequences and adapters required for cluster generation.

Consumables

- EPM (Enhanced PCR Mix)
- UDPxxxx (Nextera DNA Unique Dual (UD) Indexes)
- Microseal 'B' adhesive seals

WARNING

This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations. For additional environmental, health, and safety information, see the SDS at support.illumina.com/sds.html.

Preparation

1 Prepare the following consumables:

Reagent	Storage	Instructions
EPM	-25°C to -15°C	Thaw on ice. Vortex to resuspend. Centrifuge briefly.
UDPxxxx	-25°C to -15°C	Thaw to room temperature. Centrifuge briefly.

NOTE

Each well of the index plate contains a unique index with enough volume for one use only.

2 Assign one UDPxxxx index per library (xxxx = index primer number).

When sequencing multiple libraries on a single flow cell or flow cell lane, assign a different index to each sample library. Record sample layout orientation and the indexes for each sample library.

3 In the post-amp area, save the following I-PCR program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 50 µl
   - 98°C for 30 seconds
   - 15 cycles of:
     - 98°C for 10 seconds
     - 60°C for 30 seconds
     - 72°C for 30 seconds
   - 72°C for 5 minutes
   - Hold at 10°C

Procedure

1 Pipette to mix UDP.
Pierce the foil seal on the UD index plate with a new pipette tip for each well for only the number of samples being processed.

2 Add 10 µl index (UDPxxxx) to each sample well of the LS plate.

3 Add 20 µl EPM to each well.

4 Apply Microseal 'B' to the LS PCR plate and shake the plate at 1200 rpm for 1 minute.

5 Transfer to the post-PCR area.

6 Centrifuge the plate at 280 × g for 1 minute.

7 Place the plate on the preprogrammed thermal cycler and run the I-PCR program.

8 Relabel the plate ALS (Amplified Library Samples).

SAFE STOPPING POINT

If you are stopping, ensure that the ALS plate is sealed, and briefly centrifuge at 280 × g. Store at -25°C to -15°C for up to 7 days.

Set Up First Hybridization

During this process, a pool of oligos specific to 523 genes targeted by TruSight Oncology 500 HT hybridize to DNA and/or RNA libraries prepared in *Index PCR* on page 19. Enrichment of targeted regions requires two hybridization steps. In this step, the first hybridization, oligos hybridize to the DNA and RNA libraries overnight (8–24 hours).

Consumables

- EHB (Enrichment Hybridization Buffer)
- OPD2 (Oncology DNA Probe Pool 2)
- OPR1 (Oncology RNA Probe Pool 1)
- 96-well PCR plate
- Microseal 'B' adhesive seals

WARNING

This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations. For additional environmental, health, and safety information, see the SDS at support.illumina.com/sds.html.

About Reagents

- Use OPD2 for DNA libraries only.
- Use OPR1 for RNA libraries only.

Preparation

1 Prepare the following consumables.

Reagent	Storage	Instructions
EHB	-25°C to -15°C	Thaw to room temperature. Centrifuge briefly and then vortex to mix. Inspect for precipitates. If precipitates are present, vortex until cloudiness or crystals are dissolved.
OPD2	-25°C to -15°C	Thaw to room temperature. Vortex to resuspend. Centrifuge briefly.
OPR1	-25°C to -15°C	Thaw to room temperature. Vortex to resuspend. Centrifuge briefly.

2 If the ALS PCR plate was stored at -25°C to -15°C, perform the following steps:

a Thaw at room temperature.
b Centrifuge at 280 × g for 1 minute.
c Pipette to mix.

3 Label a new 96-well PCR plate HYB1 (Hybridization 1).

4 Save the following HYB1 program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- Set the reaction volume to 50 µl
- 95°C for 10 minutes
- 85°C for 2.5 minutes
- 75°C for 2.5 minutes
- 65°C for 2.5 minutes
- Hold at 57°C

Procedure

1 Transfer 20 µl of each library from the ALS PCR plate to the HYB1 PCR plate.

2 Add 25 µl EHB to each well.

3 Add the appropriate probe.
- For DNA libraries, add 5 µl OPD2.
- For RNA libraries, add 5 µl OPR1.

4 Apply Microseal ‘B’ to the HYB1 PCR plate and shake the plate at 1200 rpm for 2 minutes.

5 Place on the preprogrammed thermal cycler and run the HYB1 program. Hybridize for 8–24 hours (overnight) at 57°C.

Capture Targets One

This step uses SMB (Streptavidin Magnetic Beads) to capture probes hybridized to the targeted regions of interest. Three heated washes using EEW remove nonspecific DNA binding from the beads. The enriched library is then eluted from the beads and prepared for a second round of hybridization.

Consumables

- EE2 (Enrichment Elution 2)
- EEW (Enhanced Enrichment Wash)
- ET2 (Elute Target Buffer 2)
- HP3 (2 N NaOH)
- SMB (Streptavidin Magnetic Beads)
- 1.7 ml microcentrifuge tube
- 96-well MIDI plate
- 96-well PCR plate
- Microseal 'B' adhesive seals

WARNING

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About Reagents

- Make sure to use **SMB** and ***not*** **SPB** for this procedure.

Preparation

1 Prepare the following consumables:

Reagent	Storage	Instructions
EE2	-25°C to -15°C	Thaw to room temperature. Vortex to resuspend. Centrifuge briefly.
EEW	-25°C to -15°C	Thaw to room temperature. Vortex for 1 minute to resuspend.
ET2	2°C to 8°C	Bring to room temperature. Vortex to resuspend. Centrifuge briefly.
HP3	2°C to 8°C	Bring to room temperature. Vortex to resuspend. Centrifuge briefly.
SMB	2°C to 8°C	Bring to room temperature for 30 minutes. If the bead pellet is present, pipette up and down to release the pellet, and then vortex to resuspend.

2 Preheat a Hybex incubator with MIDI heat block insert to 57°C.

3 Label a new 96-well MIDI plate CAP1 (Capture 1).

4 Label a new 96-well PCR plate ELU1 (Elution 1).

Procedure

Bind

1 Remove the HYB1 PCR plate from the thermal cycler.

2 Vortex SMB for 1 minute to resuspend the beads.

3 Add 150 µl SMB to each well of the CAP1 MIDI plate.

4 Transfer 50 µl of each library from the HYB1 PCR plate to the corresponding well of the CAP1 MIDI plate.

5 Apply Microseal 'B' to the CAP1 MIDI plate and shake the plate at 1800 rpm for 2 minutes.

6 Incubate in a Hybex incubator at 57°C for 25 minutes.

7 Place on a magnetic stand for 2 minutes.

8 While on the magnetic stand, remove and discard the supernatant from each well.

Wash

1 Wash beads as follows:

   a Remove the CAP1 MIDI plate from the magnetic stand.
   b Add 200 µl EEW to each well.
   c Pipette to mix 10 times.
   d Apply Microseal 'B' and shake the plate at 1800 rpm for 4 minutes.
   If the bead pellet is still present, remove the Microseal and pipette to mix. Make sure that all beads are resuspended, and then apply a new Microseal 'B'.
   e Incubate in a Hybex incubator at 57°C for 5 minutes.
   f Place on a magnetic stand for 2 minutes.

g While on the magnetic stand, remove and discard all supernatant from each well.

2 Repeat step 1 to wash beads a **second** time.

3 Repeat step 1 to wash beads a **third** time.

4 Use a P20 pipette with fine tips to remove any residual supernatant from each well.

Elute

1 Combine the following volumes in a microcentrifuge tube to prepare the EE2+HP3 Elution Mix:

Elution Mix Component	8 Libraries (µl)	24 Libraries (µl)	48 Libraries (µl)	72 Libraries (µl)
EE2	171	512	1024	1536
HP3	9	27	55	82

2 Vortex briefly to mix.

3 Remove the CAP1 MIDI plate from the magnetic stand.

4 Carefully add 17 µl EE2+HP3 Elution Mix to each sample pellet.

5 Discard remaining elution mix after use.

6 Apply Microseal 'B' to the CAP1 MIDI plate and shake the plate at 1800 rpm for 2 minutes.

7 Place on a magnetic stand for 2 minutes.

8 Carefully transfer 15 µl eluate from each well of the CAP1 MIDI plate to the ELU1 PCR plate.

9 Add 5 µl ET2 to each eluate in the ELU1 PCR plate.

10 Apply Microseal 'B' to the ELU1 PCR plate and shake the plate at 1200 rpm for 2 minutes.

Set Up Second Hybridization

This step binds targeted regions of the enriched DNA libraries with capture probes a second time. The second hybridization ensures high specificity of the captured regions. To ensure optimal enrichment of libraries, perform the second hybridization step for 1.5–4 hours.

Consumables

- EHB (Enrichment Hybridization Buffer)
- OPD2 (Oncology DNA Probe Pool 2)
- OPR1 (Oncology RNA Probe Pool 1)
- Microseal 'B' adhesive seals

WARNING

This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations. For additional environmental, health, and safety information, see the SDS at support.illumina.com/sds.html.

About Reagents

- Use OPD2 for DNA libraries only.
- Use OPR1 for RNA libraries only.

Preparation

1 Prepare the following consumables:

Reagent	Storage	Instructions
EHB	-25°C to -15°C	Thaw to room temperature. Centrifuge briefly and then vortex to mix. If precipitates are present, vortex until cloudiness or crystals are dissolved.
OPD2	-25°C to -15°C	Thaw to room temperature. Vortex to resuspend. Centrifuge briefly.
OPR1	-25°C to -15°C	Thaw to room temperature. Vortex to resuspend. Centrifuge briefly.

2 Save the following HYB2 program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- Set the reaction volume to 50 µl
- 95°C for 10 minutes
- 85°C for 2.5 minutes
- 75°C for 2.5 minutes
- 65°C for 2.5 minutes
- Hold at 57°C

Procedure

1 Add 25 µl EHB to each well of the ELU1 PCR plate.

2 Add the appropriate probe to each well.
- For DNA libraries, add 5 µl OPD2.
- For RNA libraries, add 5 µl OPR1.

3 Apply Microseal 'B' to the ELU1 PCR plate and shake the plate at 1200 rpm for 2 minutes.

4 Place on the preprogrammed thermal cycler and run the HYB2 program. Hybridize at 57°C for 1.5-4 hours.

Capture Targets Two

This step uses SMB (Streptavidin Magnetic Beads) to capture probes hybridized to the targeted regions of interest. RSB is used to rinse the captured libraries and remove nonspecific binding from the beads. The enriched library is then eluted from the beads and prepared for sequencing.

Consumables

- EE2 (Enrichment Elution 2)
- ET2 (Elute Target Buffer 2)
- HP3 (2 N NaOH)
- RSB (Resuspension Buffer)

- SMB (Streptavidin Magnetic Beads)
- 1.7 ml microcentrifuge tube
- **[Optional]** 15 ml conical tubes
- 96-well MIDI plate
- 96-well PCR plate
- Microseal 'B' adhesive seals

WARNING

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About Reagents

- Make sure to use **SMB** and ***not*** **SPB** for this procedure.

Preparation

1 Prepare the following consumables:

Reagent	Storage	Instructions
EE2	-25°C to -15°C	Thaw to room temperature. Vortex to resuspend. Centrifuge briefly.
ET2	2°C to 8°C	Bring to room temperature. Vortex to resuspend. Centrifuge briefly.
HP3	2°C to 8°C	Bring to room temperature. Vortex to resuspend. Centrifuge briefly.
RSB	2°C to 8°C or -25°C to -15°C	Bring to room temperature. If stored at -25°C to -15°C, thaw at room temperature and vortex before use.
SMB	2°C to 8°C	Bring to room temperature for 30 minutes. If the bead pellet is present, pipette up and down to release the pellet, and then vortex to resuspend.

2 Preheat a Hybex incubator with MIDI heat block insert to 57°C.

3 Label a new 96-well MIDI plate CAP2 (Capture 2).

4 Label a new 96-well PCR plate ELU2 (Elution 2).

Procedure

Bind

1 Remove the ELU1 PCR plate from the thermal cycler.

2 Vortex SMB for 1 minute to resuspend the beads.

3 Add 150 µl SMB to each well of the CAP2 MIDI plate.

4 Transfer 50 µl of each library from the ELU1 PCR plate to the corresponding well of the CAP2 MIDI plate.

5 Apply Microseal ‘B’ to the CAP2 MIDI plate and shake at 1800 rpm for 2 minutes.

6 Incubate in a Hybex incubator at 57°C for 25 minutes.

7 Place on a magnetic stand for 2 minutes.

8 While on the magnetic stand, carefully remove and discard the supernatant from each well.

Wash

1 Wash beads as follows.

   a Remove the CAP2 MIDI plate from the magnetic stand.
   b Add 200 µl RSB to each well.
   c Apply Microseal ‘B’ to the CAP2 MIDI plate and shake the plate at 1800 rpm for 4 minutes. If the bead pellet is still present, remove the Microseal and pipette to mix. Make sure that all beads are resuspended, and then apply a new Microseal 'B'.
   d Place on a magnetic stand for 2 minutes.
   e While on the magnetic stand, use a pipette to carefully remove and discard the supernatant.

2 Use a P20 pipette with fine tips to remove any residual supernatant from each well.

Elute

1 Combine the following volumes to prepare the EE2+HP3 Elution Mix:

Elution Mix Component	8 Libraries (µl)	24 Libraries (µl)	48 Libraries (µl)	72 Libraries (µl)
EE2	209	627	1254	1881
HP3	11	33	66	99

2 Vortex to mix.

3 Remove the CAP2 MIDI plate from the magnetic stand.

4 Carefully add 22 µl EE2+HP3 Elution Mix to each sample pellet.

5 Discard remaining elution mix after use.

6 Place on a magnetic stand for 2 minutes.

7 Transfer 20 µl eluate from each well of the CAP2 MIDI plate to the ELU2 PCR plate.

8 Add 5 µl ET2 to each eluate in the ELU2 PCR plate.

9 Apply Microseal ‘B’ to the ELU2 PCR plate and shake the ELU2 PCR plate at 1200 rpm for 2 minutes.

SAFE STOPPING POINT

If you are stopping, ensure that the ELU2 plate is sealed and briefly centrifuge at 280 × g. Store at -25°C to -15°C for up to 7 days.

Amplify Enriched Library

This step uses primers to amplify enriched libraries.

Consumables

- EPM (Enhanced PCR Mix)
- PPC3 (PCR Primer Cocktail 3)
- Microseal 'B' adhesive seals

WARNING

This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations. For additional environmental, health, and safety information, see the SDS at support.illumina.com/sds.html.

Preparation

1 Prepare the following consumables.

Reagent	Storage	Instructions
EPM	-25°C to -15°C	Thaw on ice. Vortex to resuspend. Centrifuge briefly.
PPC3	-25°C to -15°C	Thaw to room temperature. Vortex to resuspend. Centrifuge briefly.

2 If the ELU2 plate was stored at -25°C to -15°C, thaw at room temperature, pipette to mix, and then centrifuge.

3 Save the following EL-PCR program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- Set the reaction volume to 50 µl
- 98°C for 30 seconds
- 18 cycles of:
  - 98°C for 10 seconds
  - 60°C for 30 seconds
  - 72°C for 30 seconds
- 72°C for 5 minutes
- Hold at 10°C

Procedure

1 Add 5 µl PPC3 to each well of the ELU2 PCR plate.

2 Add 20 µl EPM to each well.

3 Apply Microseal 'B' and shake the ELU2 PCR plate at 1200 rpm for 2 minutes.

4 Centrifuge at 280 × g for one minute.

5 Place on the preprogrammed thermal cycler and run the EL-PCR program.

Clean Up Amplified Enriched Library

This step uses SPB (Sample Purification Beads) to purify the enriched library from unwanted reaction components.

Consumables

- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- Freshly prepared 80% ethanol (EtOH)
- 96-well MIDI plate
- 96-well PCR plate
- Microseal 'B' adhesive seals

About Reagents

- Aspirate and dispense SPB slowly due to the viscosity of the solution.

Preparation

1 Prepare the following consumables:

Item	Storage	Instructions
RSB	2°C to 8°C or -25°C to -15°C	Bring to room temperature. If stored at -25°C to -15°C, thaw at room temperature and vortex before use.
SPB	2°C to 8°C	Bring to room temperature for 30 minutes.

2 Label a new 96-well MIDI plate BIND2.

3 Label a new 96-well PCR plate PL (Purified Libraries).

4 Prepare fresh 80% EtOH.

Procedure

Bind

1 Remove the ELU2 PCR plate from the thermal cycler.

2 Vortex SPB for 1 minute to resuspend the beads.

3 Add 110 µl SPB to each well of the BIND2 MIDI plate.

4 Transfer 50 µl of each library from the ELU2 PCR plate to the corresponding well of the BIND2 MIDI plate.

5 Apply Microseal ‘B’ to the BIND2 MIDI plate and shake at 1800 rpm for 2 minutes.

6 Incubate at room temperature for 5 minutes.

7 Place the BIND2 MIDI plate on magnetic stand for 5 minutes.

8 While on the magnetic stand, remove and discard all supernatant from each well.

Wash

1 Wash beads as follows.

   a Keep on magnetic stand and add 200 µl fresh 80% ethanol to each well.
   b Wait 30 seconds.
   c Remove and discard all supernatant from each well.

2 Repeat step 1 to wash beads a **second** time.

3 Use a P20 pipette with fine tips to remove residual supernatant from each well.

Elute

1 Remove the BIND2 MIDI plate from the magnetic stand.

2 Add 32 µl RSB to each well.

3 Apply Microseal 'B' and shake at 1800 rpm for 2 minutes.

4 Incubate at room temperature for 2 minutes.

5 Place on a magnetic stand for 2 minutes.

6 Transfer 30 µl of each eluate from the BIND2 MIDI plate to the corresponding well of the PL PCR plate.

SAFE STOPPING POINT

If you are stopping, apply Microseal 'B' to the PL PCR plate and briefly centrifuge at 280 × g. Store at -25°C to -15°C for up to 30 days.

Quantify Libraries (Optional)

Accurately quantify to make sure that there is sufficient library available for clustering on the flow cell. Use a fluorometric quantification method (user-supplied) to assess the quantity of enriched libraries before library normalization. Efficient bead-based library normalization requires ≥ 3 ng/µl of each library. The AccuClear Ultra High Sensitivity dsDNA Quantitation Kit has been demonstrated to be effective for quantifying libraries in this protocol.

Recommended Guidelines (AccuClear)

1 Combine 6 µl DNA standard with 44 µl RSB to dilute DNA standard to 3 ng/µl.

2 Use RSB as blank.

3 Run the diluted AccuClear DNA standard and blanks in triplicate.

4 Run libraries in single replicates.

5 Determine the average relative fluorescence unit (RFU) for DNA standard and blank.

6 Calculate the following values.
   - Average Standard RFU - Average Blank RFU = Normalized Standard RFU
   - Library RFU - Average Blank RFU = Normalized RFU for each library

Assess Quantity

Assess the resulting Normalized RFU for each library against the following criteria.

Fluorescence Measurement	Recommendation
≤ Average Blank RFU	Repeat library preparation and enrichment if purified DNA or RNA sample meets quantity and quality specifications.
> Average Blank RFU (and) < Normalized Standard RFU	Proceed to *Normalize Libraries*. Note: Using libraries with RFU below the Normalized Standard RFU might not yield adequate sequencing results to confidently call variants that can be present in the sample.
≥ Normalized Standard RFU	Proceed to *Normalize Libraries*.

Normalize Libraries

This process uses bead-based normalization to normalize the quantity of each library to ensure a uniform library representation in the sequencing pool.

Consumables

- EE2 (Enrichment Elution 2)
- HP3 (2 N NaOH)
- LNA1 (Library Normalization Additives 1)
- LNB1 (Library Normalization Beads 1)
- LNS1 (Library Normalization Storage Buffer 1)
- LNW1 (Library Normalization Wash 1)
- 1.7 ml microcentrifuge tubes
- **[Optional]** 15 ml conical tubes
- 96-well MIDI plate
- 96-well PCR plate
- Microseal 'B' adhesive seals

WARNING

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About Reagents

- Aspirate and dispense LNB1 slowly due to the viscosity of the solution.

Preparation

1 Prepare the following consumables:

Reagent	Storage	Instructions
EE2	-25°C to -15°C	Thaw to room temperature. Vortex to resuspend. Centrifuge briefly.
LNA1	-25°C to -15°C	Thaw to room temperature. Vortex to resuspend.
HP3	2°C to 8°C	Bring to room temperature. Vortex to resuspend. Centrifuge briefly.
LNB1	2°C to 8°C	Bring to room temperature for at least 30 minutes. Pipette LNB1 pellet up and down to resuspend.
LNS1	2°C to 8°C	Bring to room temperature. Vortex to resuspend.
LNW1	2°C to 8°C	Bring to room temperature. Vortex to resuspend.

2 If the PL PCR plate was stored at -25°C to -15°C, prepare it as follows.

a Thaw at room temperature.
b Centrifuge at 280 × g for 1 minute.
c Pipette to mix.

3 Label a new 96-well MIDI plate BBN (Bead-Based Normalization).

4 Label a new 96-well PCR plate NL (Normalized Libraries).

Procedure

1 Pulse vortex LNB1 tube for 1 minute at maximum speed.
Invert LNB1 tube to make sure all beads are resuspended. If a bead pellet remains, repeat vortexing step.

2 Using a P1000 pipette set at 800 µl, pipette LNB1 up and down 10 times to mix.

CAUTION
It is critical to completely resuspend the bead pellet at the bottom of the tube. Resuspension is essential to achieve consistent cluster density.

3 Combine the following reagents to create LNA1+LNB1 Master Mix:

Master Mix Component	8 Libraries (µl)	24 Libraries	48 Libraries	72 Libraries
LNA1	352	1055	2110	3166
LNB1	64	192	384	577

4 Combine the following reagents in a new microcentrifuge tube to create a fresh EE2+HP3 Elution Mix:

Elution Mix Component	8 Libraries (µl)	24 Libraries (µl)	48 Libraries (µl)	72 Libraries (µl)
EE2	304	912	1824	2736
HP3	16	48	96	144

5 Vortex to mix.

Bind

1 Vortex LNA1+LNB1 Master Mix.

2 Add 45 µl LNA1+LNB1 Master Mix to each well of the BBN MIDI plate.

3 Add 20 µl of each library from the PL PCR plate to the corresponding well of the BBN MIDI plate.

4 Apply Microseal 'B' to the BBN MIDI plate and shake at 1800 rpm for 30 minutes.

5 Place the plate on a magnetic stand for 2 minutes.

6 Remove and discard all supernatant from each well.

Wash

1 Wash beads as follows.

   a Remove the BBN MIDI plate from the magnetic stand.
   b Add 45 µl LNW1 to each well.
   c Apply Microseal 'B' and shake at 1800 rpm for 5 minutes.
   d Place on a magnetic stand for 2 minutes.
   e Remove and discard all supernatant from each well.

2 Repeat step 1 to wash a **second** time.

3 Use a P20 pipette with fine tips to remove any residual supernatant from each well.

Elute

1 Remove the BBN MIDI plate from the magnetic stand.

2 Vortex EE2+HP3 Elution Mix and then centrifuge briefly.

3 Carefully add 32 µl EE2+HP3 Elution Mix to each well.

4 Discard remaining elution mix after use.

5 Apply Microseal 'B' to the BBN MIDI plate and shake at 1800 rpm for 2 minutes.

6 Place on a magnetic stand for 2 minutes.

7 Transfer 30 µl of each eluate from the BBN MIDI plate to the corresponding well of the NL PCR plate.

8 Add 30 µl LNS1 to each library in the NL PCR plate.

9 Pipette up and down 5 times to mix.

SAFE STOPPING POINT

If you are stopping, apply Microseal 'B' to the NL PCR plate and briefly centrifuge at 280 × g. Store at -25°C to -15°C for up to 30 days.

Pool Libraries and Dilute to the Loading Concentration

1 See the denature and dilute libraries guide for your sequencing system to pool, denature, and dilute libraries to the loading concentration.

Supporting Information

Introduction

The protocol described in this guide assumes that you have reviewed the contents of this section, confirmed protocol contents, and obtained all required consumables and equipment.

Kit Contents

Make sure that you have the reagents identified in this section before proceeding to the protocol. The protocol requires one TruSight Oncology 500 HT kit and at least one IDT® for Illumina® DNA/RNA UMI UD Indexes set (A or B). Combine both sets to index 192 libraries.

Kit	Catalog #
TruSight Oncology 500 DNA/RNA High-Throughput Kit (24 Samples)	20040764
TruSight Oncology 500 DNA High-Throughput Kit (48 Samples)	20040765
TruSight Oncology 500 DNA/RNA High-Throughput Kit (72 Samples)	20040766
TruSight Oncology 500 DNA High-Throughput Kit (144 Samples)	20040767

Library prep kit plus access to the PierianDx Clinical Genomics Workspace	Catalog #
TruSight Oncology 500 DNA/RNA High-Throughput Kit (24 Samples), plus PierianDx	20040768
TruSight Oncology 500 DNA High-Throughput Kit (48 Samples), plus PierianDx	20040769
TruSight Oncology 500 DNA/RNA High-Throughput Kit (72 Samples), plus PierianDx	20040770
TruSight Oncology 500 DNA High-Throughput Kit (144 Samples), plus PierianDx	20040771

TruSight Oncology 500 DNA/RNA High-Throughput (24 Samples)

TruSight Oncology 500 DNA/RNA High-Throughput (24 Samples)

RNA Library Prep (Pre-Amp) (REF 20007000), Store at -25°C to -15°C

DNA/RNA bundle customers receive one box. DNA kit customers do not receive this box.

Quantity	Reagent	Description
1	EPH3	Elution, Primer, Fragmentation, Mix
1	FSM	First Strand Synthesis Mix
1	RT	Reverse Transcriptase
1	SSM	Second Strand Mix

Library Prep without SUA (Pre-Amp) (REF 20039147), Store at -25°C to -15°C

Quantity	Reagent	Description
2	ALB1	Adapter Ligation Buffer 1
2	EPM	Enhanced PCR Mix
2	ERA1-A	End Repair A-tailing Enzyme Mix 1
2	ERA1-B	End Repair A-tailing Buffer 1
2	LIG3	DNA Ligase 3
2	STL	Stop Ligation Buffer

Library Prep (Pre-Amp) (REF 20007003), See Storage Temperatures in Table

Quantity	Reagent	Description	Storage Temperature
1	RSB	Resuspension Buffer	2°C to 8°C or -25°C to -15°C
2	SPB	Sample Purification Beads	2°C to 8°C
1	TEB	TE Buffer	2°C to 8°C

Enrichment without TCB (Post-Amp) (REF 20040235), See Storage Temperatures in Table

Quantity	Reagent	Description	Storage Temperature
2	ET2	Elute Target Buffer 2	2°C to 8°C
2	HP3	2 N NaOH	2°C to 8°C
1	LNB1	Library Normalization Beads 1	2°C to 8°C
2	LNS1	Library Normalization Storage Buffer 1	2°C to 8°C
2	LNW1	Library Normalization Wash 1	2°C to 8°C
1	RSB	Resuspension Buffer	2°C to 8°C or -25°C to -15°C
2	SMB	Streptavidin Magnetic Beads	2°C to 8°C
2	SPB	Sample Purification Beads	2°C to 8°C

Enrichment with EHB (Post-Amp) (REF 20040234), Store at -25°C to -15°C

Quantity	Reagent	Description
3	EE2	Enrichment Elution 2
1	EEW	Enhanced Enrichment Wash
6	EHB	Enrichment Hybridization Buffer
2	EPM	Enhanced PCR Mix
1	LNA1	Library Normalization Additives 1
2	PPC3	PCR Primer Cocktail 3

DNA Probes (Post-Amp) (REF 20026138), Store at -25°C to -15°C

Quantity	Reagent	Description
1	OPD2	Oncology DNA Probes Pool 2

RNA Probes (Post-Amp) (REF 20007012), Store at -25°C to -15°C

Quantity	Reagent	Description
1	OPR1	Oncology RNA Probes Pool

TruSight Oncology 500 DNA High-Throughput (48 Samples)

TruSight Oncology 500 DNA High-Throughput (48 Samples)

Library Prep without SUA (Pre-Amp) (REF 20039147), Store at -25°C to -15°C

Quantity	Reagent	Description
2	ALB1	Adapter Ligation Buffer 1
2	EPM	Enhanced PCR Mix
2	ERA1-A	End Repair A-tailing Enzyme Mix 1
2	ERA1-B	End Repair A-tailing Buffer 1
2	LIG3	DNA Ligase 3
2	STL	Stop Ligation Buffer

Library Prep (Pre-Amp) (REF 20007003), See Storage Temperatures in Table

Quantity	Reagent	Description	Storage Temperature
1	RSB	Resuspension Buffer	2°C to 8°C or -25°C to -15°C
2	SPB	Sample Purification Beads	2°C to 8°C
1	TEB	TE Buffer	2°C to 8°C

Enrichment without TCB (Post-Amp) (REF 20040235), See Storage Temperatures in Table

Quantity	Reagent	Description	Storage Temperature
2	ET2	Elute Target Buffer 2	2°C to 8°C
2	HP3	2 N NaOH	2°C to 8°C
1	LNB1	Library Normalization Beads 1	2°C to 8°C
2	LNS1	Library Normalization Storage Buffer 1	2°C to 8°C
2	LNW1	Library Normalization Wash 1	2°C to 8°C
1	RSB	Resuspension Buffer	2°C to 8°C or -25°C to -15°C
2	SMB	Streptavidin Magnetic Beads	2°C to 8°C
2	SPB	Sample Purification Beads	2°C to 8°C

Enrichment with EHB (Post-Amp) (REF 20040234), Store at -25°C to -15°C

Quantity	Reagent	Description
3	EE2	Enrichment Elution 2
1	EEW	Enhanced Enrichment Wash
6	EHB	Enrichment Hybridization Buffer
2	EPM	Enhanced PCR Mix
1	LNA1	Library Normalization Additives 1
2	PPC3	PCR Primer Cocktail 3

DNA Probes (Post-Amp) (REF 20026138), Store at -25°C to -15°C

Customers will receive two of these boxes.

Quantity	Reagent	Description
1	OPD2	Oncology DNA Probes Pool 2

TruSight Oncology 500 DNA/RNA High-Throughput (72 Samples)

TruSight Oncology 500 DNA/RNA High-Throughput (72 Samples)

RNA Library Prep (Pre-Amp) (REF 20040222), Store at -25°C to -15°C

Quantity	Reagent	Description
3	EPH3	Elution, Primer, Fragmentation Mix
3	FSM	First Strand Synthesis Mix
3	RVT	Reverse Transcriptase
3	SSM	Second Strand Mix

Library Prep without SUA (REF 20040223), Store at -25°C to -15°C

Quantity	Reagent	Description
6	ALB1	Adapter Ligation Buffer 1
6	EPM	Enhanced PCR Mix
6	ERA1-A	End Repair A-tailing Enzyme Mix 1
6	ERA1-B	End Repair A-tailing Buffer 1
6	LIG3	DNA Ligase 3
6	STL	Stop Ligation Buffer

Library Prep (Pre-Amp) (REF 20040224), See Storage Temperatures in Table

Quantity	Reagent	Description	Storage Temperature
3	RSB	Resuspension Buffer	2°C to 8°C or -25°C to -15°C
6	SPB	Sample Purification Beads	2°C to 8°C
3	TEB	TE Buffer	2°C to 8°C

Enrichment without TCB (Post-Amp) (REF 20040225), See Storage Temperatures in Table

Quantity	Reagent	Description	Storage Temperature
6	ET2	Elute Target Buffer 2	2°C to 8°C
6	HP3	2 N NaOH	2°C to 8°C
3	LNB1	Library Normalization Beads 1	2°C to 8°C
6	LNS1	Library Normalization Storage Buffer 1	2°C to 8°C
6	LNW1	Library Normalization Wash 1	2°C to 8°C
3	RSB	Resuspension Buffer	2°C to 8°C or -25°C to -15°C
6	SMB	Streptavidin Magnetic Beads	2°C to 8°C
6	SPB	Sample Purification Beads	2°C to 8°C

Enrichment with EHB (Post-Amp) (REF 20040226), Store at -25°C to -15°C

Quantity	Reagent	Description
9	EE2	Enrichment Elution 2
3	EEW	Enhanced Enrichment Wash
18	EHB	Enrichment Hybridization Buffer
6	EPM	Enhanced PCR Mix
3	LNA1	Library Normalization Additives 1
6	PPC3	PCR Primer Cocktail 3

DNA/RNA Probes (Post-Amp) (REF 20040227), Store at -25°C to -15°C

Quantity	Reagent	Description
3	OPD2	Oncology DNA Probe Pool 2
3	OPR1	Oncology RNA Probe Pool 1

TruSight Oncology 500 DNA High-Throughput (144 Samples)

TruSight Oncology 500 DNA High-Throughput (144 Samples)

Library Prep without SUA (Pre-Amp) (REF 20040223), Store at -25°C to -15°C

Quantity	Reagent	Description
6	ALB1	Adapter Ligation Buffer 1
6	EPM	Enhanced PCR Mix
6	ERA1-A	End Repair A-tailing Enzyme Mix 1
6	ERA1-B	End Repair A-tailing Buffer 1
6	LIG3	DNA Ligase 3
6	STL	Stop Ligation Buffer

Library Prep (Pre-Amp) (REF 20040224), See Storage Temperatures in Table

Quantity	Reagent	Description	Storage Temperature
3	RSB	Resuspension Buffer	2°C to 8°C or -25°C to -15°C
6	SPB	Sample Purification Beads	2°C to 8°C
3	TEB	TE Buffer	2°C to 8°C

Enrichment without TCB (Post-Amp) (REF 20040225), See Storage Temperatures in Table

Quantity	Reagent	Description	Storage Temperature
6	ET2	Elute Target Buffer 2	2°C to 8°C
6	HP3	2 N NaOH	2°C to 8°C
3	LNB1	Library Normalization Beads 1	2°C to 8°C
6	LNS1	Library Normalization Storage Buffer 1	2°C to 8°C
6	LNW1	Library Normalization Wash 1	2°C to 8°C
3	RSB	Resuspension Buffer	2°C to 8°C or -25°C to -15°C
6	SMB	Streptavidin Magnetic Beads	2°C to 8°C
6	SPB	Sample Purification Beads	2°C to 8°C

Enrichment with EHB (Post-Amp) (REF 20040226), Store at -25°C to -15°C

Quantity	Reagent	Description
9	EE2	Enrichment Elution 2
3	EEW	Enhanced Enrichment Wash
18	EHB	Enrichment Hybridization Buffer
6	EPM	Enhanced PCR Mix
3	LNA1	Library Normalization Additives 1
6	PPC3	PCR Primer Cocktail 3

DNA Probes (Post-Amp) (REF 20040755), Store at -25°C to -15°C

Quantity	Reagent	Description
6	OPD2	Oncology DNA Probe Pool 2

TruSight Oncology 500 High-Throughput Index Kits

Each IDT for Illumina DNA/RNA UMI UD Indexes set contains two boxes.

Kit	Reference #
IDT for Illumina - UMI DNA/RNA UD Indexes Set A, Ligation (96 Indexes, 96 Samples	20034701
IDT for Illumina - UMI DNA/RNA UD Indexes Set B, Ligation (96 Indexes, 96 Samples)	20034702

IDT for Illumina - UMI DNA/RNA UD Indexes Set A, Ligation (96 Indexes, 96 Samples) (REF 20034701), Store at -25°C to -15°C

Quantity	Reagent	Description
96	UDP Set A	IDT for Illumina Nextera DNA UD Indexes Set A (96 Indexes, 96 Samples) (UDP0001-UDP0096)
96	UMI DIA	IDT for Illumina - UMI DNA Index Anchors

IDT for Illumina - UMI DNA/RNA UD Indexes Set B, Ligation (96 Indexes, 96 Samples) (REF 20034702), Store at -25°C to -15°C

Quantity	Reagent	Description
96	UDP Set B	IDT for Illumina Nextera DNA UD Indexes Set B (96 Indexes, 96 Samples) (UDP0097-UDP0192)
96	UMI DIA	IDT for Illumina - UMI DNA Index Anchors

Consumables and Equipment

Make sure that you have the required consumables and equipment before starting the protocol.

The protocol has been optimized and validated using the items listed. Comparable performance is not guaranteed when using alternate consumables and equipment.

Consumables

Consumable	Supplier
[Optional] AccuClear Ultra High Sensitivity dsDNA Quantitation Kit with 1 DNA Standard	Biotium, catalog # 31029
[Optional] AllPrep DNA/RNA FFPE Kit	QIAGEN, catalog # 80234
[Optional] QuantiFluor RNA System	Promega, catalog # E3310
[Optional] Agilent DNA 1000 Kit	Agilent, catalog # 5067-1504
[Optional] Agilent RNA 6000 Nano Kit	Agilent, catalog # 5067-1511
[Optional] Standard Sensitivity RNA Analysis Kit	Agilent, catalog # DNF-471-0500
[Optional] FFPE QC Kit	Illumina, catalog # WG-321-1001
[Optional] DNA Reference Standard	Horizon Diagnostics, catalog # HD753
[Optional] Universal Human Reference RNA	Agilent, catalog # 740000
8 microTUBE Strip (12) for LE220 and E220 *evolution*	Covaris, part # 520053
8 microTUBE-50 AFA Fiber H Slit Strip V2 (for use with ME220)	Covaris, part # 520240
Rack E220 *evolution* 8 microTUBE Strip adapter (for use with E220 *evolution*)	Covaris, part # 500430
Rack 12 place 8 microTUBE Strip adapter (for use with LE220)	Covaris, part # 500191
Rack 8 microTUBE Strip V2 (for use with ME220)	Covaris, part # 500518
1.7 ml microcentrifuge tubes, nuclease-free	General lab supplier
15 ml conical tubes	General lab supplier
50 ml conical tubes	General lab supplier
20 µl aerosol resistant pipette tips	General lab supplier
200 µl aerosol resistant pipette tips	General lab supplier
1 ml aerosol resistant pipette tips	General lab supplier
96-well storage plates, 0.8 ml (MIDI plates)	Fisher Scientific, part # AB-0859
96-well PCR plates, 0.2 ml (polypropylene)	General lab supplier
[Optional] 96-well microplate, black, flat, clear bottom	Corning, part # 3904
Nuclease-free reagent reservoirs (PVC, disposable trough)	VWR, part # 89094-658
Microseal 'B' adhesive seal (adhesive plate seal)	Bio-Rad, part # MSB-1001
RNase/DNase-free water	General lab supplier
Nuclease-free water	General lab supplier
Ethanol (200 proof for molecular biology)	Sigma-Aldrich, part # E7023

Equipment (Pre-Amp)

Equipment	Supplier
Thermal Cycler	General lab supplier
Heat block (1.5 ml microcentrifuge tube)	General lab supplier

Equipment	Supplier
(2) Heat blocks (Hybex incubator, heating base)	SciGene, catalog # • 1057-30-O (115 V) or • 1057-30-2 (230 V)
(2) MIDI heat block inserts (for use with Hybex)	Illumina, catalog # BD-60-601
Tabletop centrifuge (plate centrifuge)	General lab supplier
Microcentrifuge (1.5 ml tubes)	General lab supplier
Magnetic stand-96	Thermo Fisher, catalog # AM10027
Vortexer	General lab supplier
Plate shaker (BioShake XP)	Q Instruments, part # 1808-0505
Covaris Focused-ultrasonicator	• Covaris, part # 500219 (model LE220) or • Covaris, part # 500429 (model E220 *evolution*) or • Covaris, part # 500506 (model ME220)
[Optional] 8 microTUBE Strip Prep Station for LE220 and E220 *evolution*	Covaris, part # 500327
[Optional] Rack Loading Station (for use with ME220 micro TUBE-50 AFA Fiber H Slit Strip V2)	Covaris, part # 500523
[Optional] 2100 Bioanalyzer Desktop System	Agilent, part # G2940CA
[Optional] Fragment Analyzer Automated CE System	Agilent, part # M5310AA or M5311AA
[Optional] Spectrophotometer	General lab supplier

Equipment (Post-Amp)

Equipment	Supplier
Heat block (1.5 ml microcentrifuge tube)	General lab supplier
Heat block (Hybex incubator, 96-well plate)	SciGene, catalog # • 1057-30-O (115 V) or • 1057-30-2 (230 V)
MIDI heat block insert (for use with Hybex)	Illumina, catalog # BD-60-601
Tabletop centrifuge (plate centrifuge)	General lab supplier
Microcentrifuge (1.5 ml tubes)	General lab supplier
Magnetic stand-96	Thermo Fisher, catalog # AM10027
Vortexer	General lab supplier
Plate shaker (BioShake XP)	Q Instruments, part # 1808-0505
Thermal cycler	General lab supplier
[Optional] 2100 Bioanalyzer Desktop System	Agilent, part # G2940CA
[Optional] Fragment Analyzer Automated CE System	Agilent, part # M5310AA or M5311AA
[Optional] Spectrophotometer	General lab supplier

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Website: www.illumina.com
Email: techsupport@illumina.com

Illumina Customer Support Telephone Numbers

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Belgium	+32 80077160	+32 34002973
China	400.066.5835	
Denmark	+45 80820183	+45 89871156
Finland	+358 800918363	+358 974790110
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Spain	+34 911899417	+34 800300143
Sweden	+46 850619671	+46 200883979
Switzerland	+41 565800000	+41 800200442
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United Kingdom	+44 8000126019	+44 2073057197
Other countries	+44.1799.534000	

Safety data sheets (SDSs)–Available on the Illumina website at support.illumina.com/sds.html.

Product documentation–Available for download from support.illumina.com.

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